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Immune-Adherence with Soluble Antigens

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Summary. Immune-adherence has been demonstrated by haemagglutination with four soluble antigens, two polysaccharide and two protein.

A zone phenomenon occurs with the immune-adherence of soluble antigens similar to that found with other antigen-antibody reactions.

It is suggested that the failure of immune-adherence with two soluble antigens, despite marked fixation of complement, is due to the failure of the antigen-antibody complexes to fix all four components of complement.

INTRODUCTION

THE adhesion of antigens to human and primate red cells in the presence of antibody and complement was first described with trypanosomes (Duke and Wallace, 1930; Wallace and Wormall, 1931; Brown and Broom, 1938). A similar adhesion of trypanosomes to animal platelets was described by Rieckenberg (1917). The adhesion of bacteria to human and primate erythrocytes was described by Nelson (1953) during studies on the phagocytosis of *Treponema pallidum*. He showed that this phenomenon, which he called immune-adherence, only occurred in the presence of antibody and complement. His study was extended to other bacteria, e.g. *Diplococcus pneumoniae*, *Shigella paradysenteriae*, *Salmonella typhi*, *Mycobacterium tuberculosis* and *Staphylococcus aureus*. Other particulate antigens large enough to be seen under the light microscope have also been shown to undergo immune-adherence. These include rickettsiae, vaccinia virus, starch grains and zymosan (Nelson, 1956; 1957).

In an experiment based upon the findings of Mayer, Levine, Rapp and Marucci (1954), who defined the sequence of combination of the components of guinea pig complement to sensitized sheep erythrocytes, Nelson (1956) suggested that the sequence of combination of complement components to sensitized starch to produce immune-adherence occurred in the same three steps. He therefore postulated by analogy with the findings of Mayer that the first step involved the fixation of the first component (C′1) and the fourth component (C′4), the second step the fixation of the second component (C′2), and the final step the reaction with the third component (C′3).

As the haemagglutination patterns produced by immune-adherence of bacteria were found to be directly related to the number of erythrocytes with adherent antigenic particles, the immune-adherence of antigens not directly visible under the light microscope could be investigated. This work was extended to viruses, especially phage T2 labelled with ^{35}S, by Taverne (1957), who showed a direct relation between the radioactivity on the surface of erythrocytes and the haemagglutination patterns produced by immune-adherence of the phage.

The present work intends to show that immune-adherence is a generalized immunological phenomenon involving complement. Soluble antigens are shown to take part in

immune-adherence as well as particulate antigens. A zone phenomenon, comparable to that found with complement fixation, can also be demonstrated in the region of antigen excess. The failure of certain antigen-antibody complexes to immune-adhere despite fixing complement is noted and an attempt made to explain this failure.

MATERIALS

GLASSWARE

All glassware was acid cleaned.

DILUENTS

Veronal buffered saline containing Ca^{++} and Mg^{++} was prepared according to Fulton and Dumbell (1949). For immune-adherence experiments this diluent was used with the addition of 0·5 per cent crystalline bovine albumin (Armour).

ERYTHROCYTES

Human blood was obtained from the blood bank in acid citrate dextrose solution, washed three times in veronal buffered saline and made up to a 1·5 per cent solution in veronal buffered saline with bovine albumin for immune-adherence.

Sheep blood for complement fixation tests was stored in Alsever's solution.

COMPLEMENT

Guinea pig complement was prepared by bleeding eight guinea pigs by cardiac puncture and storing the pooled sera at $-70°$.

Reagent 2 for the detection of $C'2$ was prepared according to Kabat and Mayer (1948) and consisted of the mid-piece fraction of guinea pig complement with an equal volume of heated guinea pig serum. It was titrated before use to check complete removal of the missing component, and for its ability to detect this component in the dilution to be used.

ANTIGENS

Egg Albumin

A stock 1 per cent solution of egg albumin was prepared in 0·9 per cent saline from dried egg albumin (Gurr) and filtered through a sintered glass filter. Dilutions were made from this stock.

Diptheria Toxoids

Toxoids RXM 4813/BA 2759 (150 Lf units/ml.) and BJG 291/3 purified (5500 Lf units/ml.) were kindly supplied by the Wellcome Research Laboratories, Beckenham.

Bacterial Polysaccharides

Bacterial polysaccharides from *Shigella flexneri* 4A and *Salmonella typhi* 0901 were prepared by the method of Boivin, Mesrobeanu and Mesrobeanu (1933), by extraction with

trichloracetic acid. The extracts were dialysed to remove all traces of trichloracetic acid and filtered through sintered glass filters. They were stored at $-70°$.

Streptococcal Group A Polysaccharide

This was prepared by the formamide extraction method of Fuller (1938).

ANTISERA

Egg Albumin, Shigella flexneri 4A, Salmonella typhi

Antisera against egg albumin, *S. flexneri* 4A and *S. typhi* were prepared in rabbits by giving an intravenous injection three times a week of 1 ml. of a 1 per cent egg albumin or a heat-killed bacterial suspension containing 10^9 bacteria per ml. A three-week course was given and the animals were then rested ten days before being bled from an ear vein.

Diphtheria Antitoxins

Diphtheria antitoxins of low potency were prepared by injecting 1 ml. of toxoid RXM 4813/BA 2759 intramuscularly into the flank of a rabbit and three guinea pigs, three times a week for three weeks. After ten days' rest the rabbit was bled from an ear vein and the guinea pigs by cardiac puncture. The sera from the three guinea pigs were pooled.

The potency of the two sera was tested by neutralization of toxin injected intracutaneously into the back of guinea pigs. The rabbit serum was found to contain 3 units of antitoxin/ml. and the guinea pig antitoxin 5 units of antitoxin/ml.

METHODS

IMMUNE-ADHERENCE REACTIONS

All experiments on immune-adherence were made by observing red cell agglutination patterns in reaction mixtures in Kahn tubes.

In each of a series of tubes 0·2 ml. of a dilution of antiserum and 0·2 ml. of a dilution of complement were added to 0·5 ml. of a dilution of antigen. All dilutions were made in veronal buffered saline with added bovine albumin. Guinea pig serum was used as a source of complement at a dilution of 1/32, which produced ++++ immune-adherence haemagglutination patterns with optimum antigen-antibody mixtures, and did not contain demonstrable natural haemagglutinins against human red cells. The tubes were shaken vigorously by hand and were then incubated in a waterbath at 37° for 20 minutes. 0·1 ml. of 1·5 per cent human erythrocytes were added and the tubes shaken again. The tubes were replaced in the waterbath and the red cells allowed to settle. The patterns formed by the cells settling could be distinguished after 60–70 minutes. Later readings were sometimes difficult because the agglutinated cells tended to slip to the bottom of the tube and so spoil their patterns.

Haemagglutination due to the immune-adherence of soluble antigens was so weak that agitation of the tube would completely break up the pattern. The act of tranferring the cellular deposit to the microscope slide would usually tend to break up any clumps of red cells formed by immune-adherence.

The following kinds of haemagglutination patterns were recognized:

(1) ++++: all the cells formed a layer over the bottom of the tube, extending a short way up the sides. There was no trace of a central button of cells;
(2) +++: the cells still formed a layer but with some crenation of its edge;
(3) ++: there was still a definite but thinner layer spreading up the side of the tube, most of the cells forming a central disc;
(4) +: the cells formed a compact central button with occasional flakes of agglutinated cells visible surrounding it;
(5) −: a compact button or ring of cells with no visible agglutination surrounding it.

Controls

In each experiment it was necessary to show that none of the sera contained natural agglutinins against human red cells or, if these were present at the dilutions used, to absorb them out before the serum was used. Controls were also put up to assure that there was no immune-adherence due to soluble antigens in the sera used. In every case it was shown that haemagglutination would not take place in the absence of complement, and that the reaction did not take place with sheep erythrocytes. It was also necessary to exclude natural antibodies to the test antigen in the complement serum.

COMPLEMENT FIXATION TESTS

The colorimetric method of Kabat and Mayer (1948), using one 50 per cent haemolytic-unit of complement, was used throughout, except in the comparison of the immune-adherence and fixation of complement by egg albumin and its antiserum, when a three-dimensional titration of antigen, antibody and complement was performed using the micro method of Fulton and Dumbell (1949). In addition the Fulton and Dumbell method was used as a quantitative assessment of the maximum amount of whole complement fixed by the streptococcal group A polysaccharide and diphtheria toxoid BJG 291/3. Fixation in all cases was at 4° overnight. .

DETECTION OF FIXATION OF C′2

Fixation of whole complement and C′2 were demonstrated respectively in two similar series of reaction mixtures, using 0·2 ml. volumes, which contained one 50 per cent haemolytic unit of complement (1/100 guinea pig serum), kept overnight at 4° for full fixation of complement (Kabat and Mayer, 1948). Complement fixation was shown in one series in the usual way, by adding sensitized sheep red cells; in the second series R2, that is, 1/100 guinea pig mid-piece and 1/100 heated guinea pig serum, each in 0·2 ml. volume, was added at the same time as the sensitized cells. Results were read colorimetrically.

Controls showed that the primary antigen-antibody and complement complex still did not fix any more of the C′1 and C′4 in the R2.

RESULTS

(1) BACTERIAL POLYSACCHARIDES

The immune-adherence due to the polysaccharides prepared from *S. flexneri* 4A and *S. typhi* 0901 is shown in Tables 1 and 2, where ten-fold dilutions of antigen from 10^{-1} to 10^{-7} are titrated against two-fold dilutions of antiserum from 1/8 to 1/1024. Both antigens show marked prozones with an optimum antigen dilution at 10^{-3}. In the case of the

S. typhi polysaccharide, the zone of immune-adherence is very narrow and would therefore have been missed if a range of antigen dilutions had not been used.

TABLE 1

HAEMAGGLUTINATION DUE TO IMMUNE-ADHERENCE OF *Shigella flexneri* 4A POLYSACCHARIDE

Antiserum dilutions	*Antigen dilutions*						
	10^{-1}	10^{-2}	10^{-3}	10^{-4}	10^{-5}	10^{-6}	10^{-7}
1/8	—	++	++++	++++	+++	++	—
1/16	—	++	++++	++++	++	+	—
1/32	—	++	++++	+++	++	+	—
1/64	—	++	++++	+++	+	—	—
1/128	—	++	+++	++	—	—	—
1/256	—	++	++	+	—	—	—
1/512	—	++	++	+	—	—	—
1/1024	—	—	+	—	—	—	—

TABLE 2

HAEMAGGLUTINATION DUE TO IMMUNE-ADHERENCE OF *Salmonella typhi* 0901 POLYSACCHARIDE

Antiserum dilutions	*Antigen dilutions*						
	10^{-1}	10^{-2}	10^{-3}	10^{-4}	10^{-5}	10^{-6}	10^{-7}
1/8	—	—	++++	+++	++	+	—
1/16	—	—	++++	++	+	—	—
1/32	—	—	++++	—	—	—	—
1/64	—	—	+++	—	—	—	—
1/128	—	—	++	—	—	—	—
1/256	—	—	+	—	—	—	—
1/512	—	—	—	—	—	—	—
1/1024	—	—	—	—	—	—	—

Immune-adherence was not observed with steptococcal group A polysaccharide and rabbit antiserum over a wide range of antigen and antibody dilutions, despite precipitation in optimum antigen-antibody mixtures and fixation of twelve 50 per cent haemolytic units of complement in a Fulton and Dumbell test (1949). Fixation of complement overnight at 4° followed by half an hour at 37° did not induce immune-adherence.

TABLE 3

HAEMAGGLUTINATION DUE TO IMMUNE-ADHERENCE OF DIPHTHERIA TOXOID RXM 4813/BA 2759 AND RABBIT ANTITOXIN (3 UNITS/ML.)

Antitoxin dilutions	*Toxoid dilutions (Lf units/ml.)*								
	37·5	*18·25*	*9·13*	*4·56*	*2·28*	*1·14*	*0·57*	*0·28*	*0·14*
1/8	—	+++	++++	++++	++++	++++	+++	+	—
1/16	—	+++	++++	++++	++++	++++	+++	++	—
1/32	—	++++	++++	++++	++++	++++	+++	++	+
1/64	—	++++	++++	++++	++++	++++	++++	+++	+
1/128	—	++++	++++	++++	+++	+++	++	—	—
1/256	—	—	+++	++++	+++	+++	—	—	—
1/512	—	—	—	++	++	—	—	—	—
1/1024	—	—	—	—	—	—	—	—	—

(2) DIPHTHERIA TOXOIDS

Table 3 shows the haemagglutination patterns produced in a titration by immune-adherence of two-fold dilutions of diphtheria toxoid RXM 4813/BA 2759 against two-fold dilutions of rabbit antitoxin (3 units/ml.). As with the bacterial polysaccharides there was a marked prozone. The optimum antigen dilution corresponded well with that expected in a toxoid antitoxin reaction and lay between 4·56 and 2·28 Lf units of toxoid per ml. Similar results were found with a guinea pig antitoxin (5 units per ml.) when the zone of optimum antigen dilutions lay around 5 Lf units/ml.

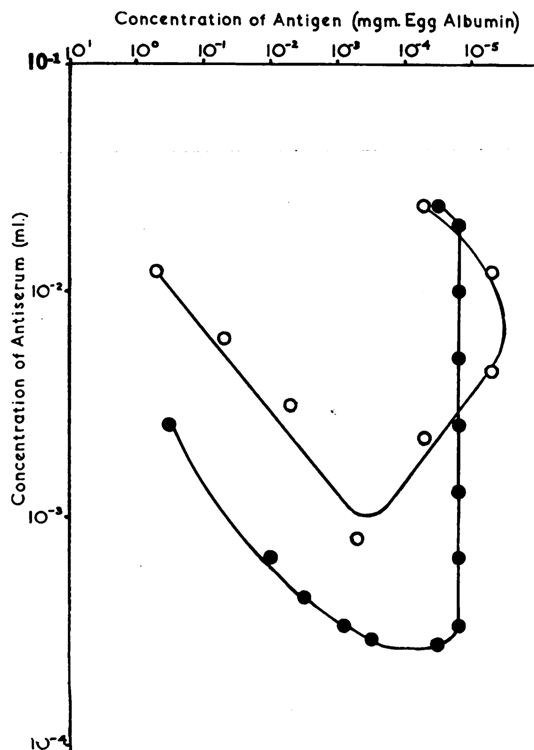

FIG. 1. Comparison of the complement contours of the immune-adherence reaction, and haemolytic complement fixation test of the egg albumin anti-egg albumin system, plotted as final concentrations in the reaction mixtures. The concentrations plotted are the final concentrations.
—O—O— Immune-adherence
—●—●— Haemolytic complement fixation

Immune-adherence was not found with diphtheria toxoid BJG 291/3 and the rabbit antiserum despite a maximal fixation of five 50 per cent haemolytic units of complement, in a Fulton and Dumbell (1949) test.

(3) EGG ALBUMIN

Table 4 shows the haemagglutination patterns produced by the immune-adherence of egg albumin with a rabbit antiserum. Ten-fold dilutions of the stock 1 per cent egg

albumin were titrated in a chess-board against two-fold dilutions of antiserum. There was again a marked prozone in the area of antigen excess. The optimum antigen dilution was 10^{-4} of the stock 1 per cent solution.

The complement fixation curve shown in Fig. 1 represents the antigen and antiserum dilutions which give 50 per cent haemolysis with one 50 per cent haemolytic unit of complement. The data on which this curve was drawn were derived from seven constant antigen chess-board titrations of antiserum against complement by the method of Fulton and Dumbell (1949). Seven constant antigen and eight constant antiserum curves were plotted and the points on Fig. 1 were taken as the antigen and antiserum dilutions at which these graphs crossed the line representing one 50 per cent haemolytic unit of

TABLE 4

HAEMAGGLUTINATION DUE TO IMMUNE-ADHERENCE OF EGG ALBUMIN

Antiserum dilutions	*Antigen dilutions*						
	10^{-1}	10^{-2}	10^{-3}	10^{-4}	10^{-5}	10^{-6}	10^{-7}
1/8	++	+++	++++	++++	+++	—	—
1/16	++	++	++++	++++	++++	++	—
1/32	—	++	++	++++	++++	+++	—
1/64	—	—	++	++++	++++	+	—
1/128	—	—	—	+++	+	—	—
1/256	—	—	—	++	—	—	—
1/512	—	—	—	—	—	—	—
1/1024	—	—	—	—	—	—	—

complement. The immune-adherence curve was derived from the ++ haemagglutination readings in Table 4; the final concentrations of antigen and antiserum were on the same scale as those in the complement fixation contour. With the egg albumin system used, prozones and optimal proportions in the two tests are similar, in spite of differences in fixation time and temperature, and in the doses of complement used in the two tests, although the antiserum/antigen ratio is definitely higher in immune-adherence. This observation is compatible with the view that the two reactions are dependent on a similar fixation of complement. More antibody appears to be needed for immune-adherence than for complete complement fixation with the amount of complement used.

Possible Explanation for the Failure to obtain Immune-Adherence with every Soluble Antigen

Failure of immune-adherence despite fixation of complement was found with two of the soluble antigens investigated. These were streptococcal group A antigen prepared by the method of Fuller (1938) with rabbit antiserum, and diphtheria toxoid BJG 291/3 with rabbit antiserum (3 units of antitoxin/ml.).

Nelson (1956) showed that the fixation of complement, necessary before immune-adherence could take place, needed a temperature of 37° and the presence of both Ca^{++} and Mg^{++} ions. He therefore suggested, by analogy with the work of Mayer *et al.* (1954), that all four components of complement were required for induction of reactivity in immune-adherence. Inhibition of immune haemolysis in the haemolytic complement fixation test theoretically needs the fixation of only one of the four components of complement. It was therefore thought necessary to see whether less than the full four components

of complement had been fixed in those cases where complement fixation occurred but no immune-adherence could be deomonstrated.

As Mayer *et al.* (1954) had shown in immune haemolysis that C′4 and C′1 were the first components to be fixed and were fixed together, it was thought that some failure to fix complement components might occur after this stage. As C′2 was the component fixed in the second stage, it was decided to see whether C′2 was reduced in the reaction mixture after fixation had been allowed to proceed at 4° for eighteen hours in systems which showed immune-adherence.

Though the difficulties of detecting fixation of C′2 have been stressed by Heidelberger, Jonsen, Waksman and Manski (1951), fixation of C′2 was detected in three systems which had been shown to immune-adhere, in reaction mixtures containing one 50 per cent haemolytic unit C′. These systems were egg albumin, diphtheria toxoid RXM 4813/BA 2759,

TABLE 5

FIXATION OF COMPLEMENT AND IMMUNE-ADHERENCE WITH SOLUBLE ANTIGENS WHICH DO AND DO NOT IMMUNE-ADHERE

(1) *Egg albumin*

	Antiserum dilutions (Antigen constant—dilution 10^{-4} *percent)*							
	1/16	1/32	1/64	1/128	1/156	1/512	1/1024	1/2048
Immune-adherence ..	++++	++++	++++	+++	++	—	—	—
Complement not fixed (% lysis)	0	0	0	0	0	0	0	50
% lysis with R2 added to detect residual C′2	0	0	0	0	0	0	25	50

(2) *Streptococcal polysaccharide*

	Antiserum dilutions (Antigen constant—dilution 1/20)				
	1/32	1/64	1/128	1/256	1/512
Immune-adherence	—	—	—	—	—
Complement not fixed (% lysis)	0	0	0	25	50
% lysis with R2 added to detect residual C′2	50	50	50	50	50

(3) *Diphtheria toxoid BJG 291/3*

	Concentration of toxoid (Lf units/ml.) (Rabbit antiserum constant—dilution 1/20)										
	18	*9·0*	*4·5*	*2·25*	*1·125*	*0·56*	*0·28*	*0·14*	*0·07*	*0·035*	*0·017*
Immune-adherence	—	—	—	—	—	—	—	—	—	—	—
Complement not fixed (% lysis) ..	0	0	0	0	0	0	0	0	0	10	25
% lysis with R2 added to detect residual C′2	50	50	50	50	50	50	50	50	50	50	50

Results of complement fixation expressed as % hæmolysis in the presence of one 50 % hæmolytic unit of complement.

and *S. typhi* O antigen, all with rabbit antisera. The results with egg albumin are shown in the first part of Table 5, where egg albumin is used as antigen at its optimum dilution 10^{-4} of the stock 1 per cent solution. The residual complement was measured in mixtures containing a constant amount of antigen and progressive dilutions of antiserum, and the C′2 activity was measured in the reaction mixture after complement had been fixed. No haemolysis was demonstrable after the addition of R2 with the sensitized sheep cells over the whole range of antiserum dilutions over which complement was fixed, apart from the last dilution.

Failure of fixation of the C′2 in one unit of C′ was shown with streptococcal group A polysaccharide and its antiserum. The polysaccharide was used in a dilution of 1/20 and was titrated by complement fixation with dilutions of antiserum down to 1/512. No haemolysis was observed at antiserum dilutions down to 1/128, 25 per cent at 1/256 and full 50 per cent haemolysis was observed with an antiserum dilution of 1/512.

When reagent R2 was added at the same time as the sensitized red cells 50 per cent haemolysis was noted up to a dilution of 1/32 (more concentrated serum could not be used as it was found to be anticomplementary above this dilution).

Similar failure of fixation of the C′2 in one unit of C′ was found in the reaction between diphtheria toxoid BJG 291/3 and rabbit antiserum, which did not immune-adhere, although diphtheria toxoid RXM 4813/BA 2759, which immune-adhered, had been found to fix C′2. Dilutions of toxoid BJG 291/3 were titrated with a constant dilution (1/20) of rabbit antiserum. Fixation of complement occurred down to a concentration of 0·035 Lf units toxoid/ml. The addition of R2 with the sensitized red cells produced 50 per cent lysis throughout.

DISCUSSION

The finding that soluble antigens as well as particulate antigens will undergo immune-adherence increases the range of antigens with which immune-adherence can be demonstrated to one comparable with that of antigens which fix complement.

Soluble antigens in the presence of constant antiserum give little or no immune-adherence at high antigen concentration, maximum immune-adherence at lower concentration and no immune-adherence at even lower concentration. The pattern of zoning is similar to that found in complement fixation and other antigen-antibody reactions. This finding has not been noted in previous work (Taverne, 1957) because the antigens used could not be employed in high enough concentration to inhibit the reaction.

The failure of some soluble antigens to immune-adhere despite fixing complement may be due to the fixation of less than four components of complement. In two such cases it has been possible to demonstrate an apparent failure of fixation of a component of complement (C′2) after a large amount of complement has been fixed as shown by the haemolytic complement fixation test, whereas this could not be demonstrated with systems with which similar amounts of complement had been fixed but immune-adherence had occurred. The quantitative estimation of C′2 is difficult as Heidelberger *et al.* (1951) have indicated. It is therefore not justifiable to claim that the observed difference in fixation of C′2 necessarily accounts for the observed differences in immune-adherence, which have appeared to be correlated with it. On the other hand, the correlation of these differences is consistent with the view that failure of immune-adherence in the system studied was due to failure of sufficient fixation of C′2.

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